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J. Infect Dis (1982)146(6)pp819-825 Williams et al.
Antimicrob. Agents. Chemother (1980)18(3) p409-415 Gatmaitan et al.

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(54) Preparation of nucleic acid derivatives

(57) A method of producing double-stranded nucleic acid derivatives having RNA as the parent body (the total molecular size distribution of which is from 4S to 13S expressed as sedimentation constant value of, alternatively, is such that the derivatives have base numbers in the range 50 to 10,000) including sizing nucleic acid polymers and subsequently combining them. Before combination the sized polymers may be subjected to ion-exchange treatment (to effect a size determination). Such treatment with ion-exchange will also serve to remove pyrogens from nucleic acid derivatives intended for administration by injection.

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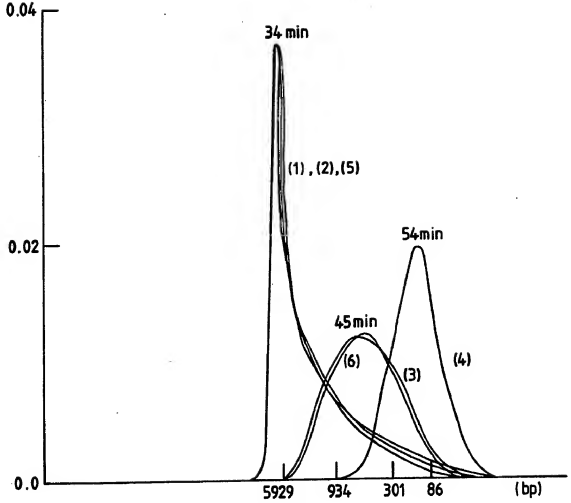

FIG.1.

PREPARATION OF NUCLEIC ACID DERIVATIVES AND MEDICAL COMPOSITIONS CONTAINING THEM

The present invention relates to a method for the preparation of nucleic acid derivatives and to the preparation of medical compositions containing such derivatives.

Nucleic acids are composed of purine rings or pyrimidine rings and ribose, or like sugars, bonded to the rings, the constitutional elements being linked to each other via phosphate bridges to form a chain structure.

Among the nucleic acids, RNA (ribonucelotide polymer) is a chain like macromolecular compound having ribose as a sugar in which the sugar moieties are bonded to each other via a phosphate bridge by diester bonds. In double-stranded nucleic acids, the purine or pyrimidine ring moieties of the nucleic acid-forming bases (for example, inosine, adenosine, cytidine, uridine, etc.) are bonded by a so-called complimentary hydrogen bond to give a double helix stereo-structure. Since nucleic acids of double-stranded structure are expected to have useful physiological functions, numerous studies have been carried out on them (Biochemical and Biophysical Research Communications, 58, 1974, etc.).

Among such nucleic acids, a synthetic double-stranded RNA polyinosinic acid·polycytidylic acid derivative is hereinafter referred to as a "poly-I·poly-C" derivative; and polyinosinic acid, which is a constitutional moiety of the derivative, is referred to as "p ly-I" and the polycyitdylic acid as "poly-C".

Various natural and synthetic double-stranded RNA's are known to have an interferon-inducing ability (Field et al, Proc. Nat. Acad. Sci., U.S., 58, 104, 1967; Field et al, Proc. Nat. Acad. Sci., U.S., 58, 2102, 1967, Field et al, Proc. Nat. Acad. Sci., U.S., 61, 340, 1968, Tytell et al, Proc. Nat. Acad. Sci., U.S., 58, 1719, 1967; Field et al, J. Gen. Physiol., 56, 905, 1970; De Clercq et al, Methods of Enzymology, 78, 291, 1981).

Typical examples of known synthetic nucleic acid derivatives are mentioned below.

Synthetic Nucleic Acid Derivatives for Interferon-Inducers

(I) Homopolymer·Homopolymer Complexes (Double-stranded nucleic acid polymers having poly-I·poly-C as the parent structure)

(1) Base-Modified Derivatives:

Polyinosinic acid·poly(5-bromocytidylic acid);

Polyinosinic acid·poly(2-thiocytidylic acid);

Poly(7-deazainosinic acid)·polycytidylic acid;

Poly(7-deazainosinic acid)·poly(5-bromocytidylic acid).

(2) Sugar-Modified Derivatives:

Poly(2'-azidoinosinic acid)·polycytidylic acid.

(3) Phosphoric Acid-Modified Derivatives:

Polyinosinic Acid·poly(cytidine-5'-thiophosphoric acid).

(II) Intermodified Copolymers:

Poly(adenylic acid-uridylic acid).

(III) Homopolymer·Copolymer Complexes:

Polyinosinic acid·poly(cytidylic acid, uridylic acid);

Polyinosinic acid·poly(cytidylic acid, 4-thiouridylic acid).

(IV) Synthetic Acid/Polycation Complexes:

Polyinosinic acid·polycytidylic acid·poly-L-lysine (hereinafter referred to as "poly-ICLC").

(V) Others:

Polyinosinic acid·poly(1-vinylcytidylic acid).

As noted above, various double-stranded RNA's especially derivatives comprising poly-I·poly-C as the parent body, have been reported in recent years. There is an established theory on a series of nucleic acid derivatives including them with respect to the relationship between the structure of the derivatives and their functions (De Clercq et al, Texas Reports on Biology and Medicine, 41, 77, 1982).

We have found, on the basis of the above-mentioned prior art, that when poly-I·poly-C and various derivatives thereof having poly-I·poly-C moiety as the parent body are sized so that the whole molecular size distribution of the derivative is within the range of from 4S to 13S as the sedimentation constant value thereof (or from about 50 to 10000 or so as the number of bases in the derivatives), the thus sized derivatives have a noticeably lowered toxicity and still have the physiological activities mentioned below. Accordingly, we filed Japanese Patent Application No. 62-167433 and a corresponding application claiming priority therefrom.

In addition to the above-mentioned study, we further investigated various methods for efficiently obtaining such products. Specifically, we have variously investigated a method of sizing nucleic acid derivatives so to give a molecular size distribution of from 50 to 10,000 or so as the number of bases thereof and a method of forming a double-stranded nucleic acid polymer from two different sorts of single-stranded nucleic acid polymer. With regard to the first method, the operation of restricting the molecular size distribution of nucleic acid derivatives to a determined range is herein referred to as "sizing". Since sizing is accompanied by conversion into low molecular substances in accordance with the method of the present invention, sizing includes "chain-shortening". The second method is referred to as "annealing" hereinafter. The base pair (hereinafter referred to as "bp") is generally used as the unit for representing the molecular size of nucleic acids and may be used for representing the molecular size of nucleic acids by way of the number of the bases constituting the nucleic acid. (For instance, 10 bp means a double-stranded polymer having 10 bases.) In the present specification, nucleic acid polymers other than double-stranded polymers are also referred to and, therefore, the term "base number" (number of bases) is used herein in place of "bp" for representing the molecular size of nucleic acids. (For instance, a nucleic acid polymer having a "base number" of 10 is one that contains 10 bases.

When the molecular size of a nucleic acid is to be determined or identified, the so-called sedimentation constant value (S value) is generally and widely used. However, we could obtain the above-mentioned base number of nucleic acids by high performance liquid chromatography (HPLC) using a gel-filtration column or electrophoresis (discussed below in detail) in which double-stranded DNA's (M13 phage-DNA fragments) having known molecular sizes are used as markers and the base number of the nucleic acid to be determined is calculated on the basis of the base number of the control.

Hitherto, the sedimentation constant value (S value) has widely been utilized for representation of the molecular weight of macromolecular nucleic acid substances including commercially available substances. However, because of the progress of experimental techniques in recent years, more accurate means for determining the molecular weight of macromolecular substances have been established by the use of gel electrophoresis, gel filtration chromatography, ion-exchange chromatography or the like so that the determination of the chain length of macromolecular nucleic acid substances has become possible. In this situation, the relation between the representation by the S value and the representation by chain length could become problematic. In particular, since the respective nucleic acid molecules have their intrinsic values herein represented by and S value, there is not always any problem on the point as to whether or not the representation by S value and the representation by chain length could accurately correspond with each other as means f representing the molecular weight of nucleic acids.

Accordingly, for the presentation of the molecular weight of the nucleic acid polymers of the present invention, representation by an S value is also employed in the description of the present specification in accordance with the conventional method in the field of nucleic acid chemistry. However, since the "S value" is one obtained by measuring the molecular weight of macromolecular nucleic acid substances in the form of a molecular mass as a whole (or in the form of a molecular state of the substance), the representation on the basis of the measurement of the chain length of the substance (the "base number" herein referred to) is also used together with the "S value". This is especially because the boundary in the molecular weight distribution is required to be more definitely represented in the embodiment of the present invention.

In accordance with conventional means of sizing poly-I·poly-C and various derivatives thereof having the said poly-I·poly-C as the parent body to give sized double-chain nucleic acid polymers, already existing double-chain nucleic acid polymers are decomposed into lower molecular compounds, or alternatively, single-stranded nucleic acids are hydrolyzed into low molecular compounds before annealing. However, the conventional methods are not convenient for obtaining the intended product on an industrial scale since the sizing requires a long time and the process cannot be performed rapidly. In addition, the conventional means are not always satisfactory from the view point of the yield of the products.

On the other hand, if single-stranded nucleic acid polymers are, after sizing, required to be sulfurized, the polymers are sulfurized with hydrogen sulfide and then the hydrogen sulfide is evaporated off from the solvent, in accordance with a conventional method. Namely, pyridine is evaporated out from the reaction solution, after sulfurization, for example, with a vacuum pump so that hydrogen sulfide may be removed from the reaction solution together with pyridine. However, hydrogen sulfide vaporizes in air, and therefore, the performance of the said method on an industrial scale is disadvantageous from the viewpoint of environmental pollution. In addition, in this method, the aqueous layer separated after evaporation of the pyridine is dialysed against running water to give the intended product. As a result, this method requires at least 3 days for complete operation and the yield of product is at most 80% or so. Thus the conventional method has various technical problems from the standpoint of yield, cost and operation time.

The sizing technique itself could not be said to be of no trouble in the prior art.

In this technical field, it is common for a nucleic acid polymer to be heated in the presence of formaldehyde to hydrolyze it into low molecular compounds. In this method, products with a desired chain length are obtained by appropriately controlling the reaction time and reaction temperature, and then subjecting the reaction solution to dialysis so as to remove over decomposed compounds having too low a molecular size. In accordance with the

conventional method, however, compounds with various different molecular weight distributions would be formed by sizing, even though the reaction conditions are kept constant, in accordance with the properties of the nucleic acid polymers used. Therefore, the method has reproducibility problems. It is believed that the reason is because, as the raw materials for use in the method are prepared by an enzyme reaction, the size of the raw materials cannot be maintained constant. In addition, by using dialysis it is in principle impossible to remove nucleic acid polymers having a longer chain length that the products formed. Under the circumstances, a method of overcoming the above-mentioned problems is desirable.

In accordance with a first embodiment of the invention there is provided a method of producing double-stranded nucleic acid derivatives having RNA as the parent body, the whole molecular size distribution of which falls within the range of from 4S to 13S measured as sedimentation constant value, in which the nucleic acid polymers are sized prior to being annealed.

The invention also provides a method of producing double-chain nucleic acid derivatives having RNA as the parent body, in which the molecules for the maximum distribution in the total molecular size distribution of the derivatives have a base number of from 50 to 10,000, in which nucleic acid polymers are sized prior to being annealed.

More specifically, the invention covers processes including the following points:

(1) single-chain nucleic acid polymers are sized prior to being annealed;

(2) For the sizing in the step (1), HPLC (gel filtration high performance liquid chromatography) is employed in place of conventional electrophoresis so that the molecular size distribution of the products is numerically defined. Accordingly, the fluctuation of the molecular size distribution can easily be checked so that products having the intended molecular size distribution range of from 4S to 13S as sedimentation constant value (or from 50 to 10,000 or so as base number) can rapidly be obtained. The rapid and accurate process of selecting the products with the desired chain length has been established by the present invention.

(3) After sizing, a lower alcohol may be added to the reaction solution to isolate the products. (In conventional methods, the products are obtained by dialysis.) We have established a very simple isolation step to increase the product yield.

(4) After sizing, if the sized single-chain nucleic acid polymers are to be sulfurized, the polymers are first sulfurized with hydrogen sulfide and then, after an aryl alcohol has been added to the sulfurization reaction solution, the resulting solution is subjected to centrifugation so as to remove the hydrogen sulfide. (In conventional methods, the hydrogen sulfide is directly

evaporated from the solvent.) Thus the present invention includes a simple hydrogen sulfide-removing step to increase the product yield.

(5) As a method of controlling and restricting the molecular weight distribution of the sized single-stranded nucleic acid polymers, an ion-exchange resin is employed. (This operation is herein referred to as "size-restriction".)

Annealing is a step of binding complementary single-stranded nucleic acid polymers into a double-stranded polymer, and this is an operation which can naturally be performed with ease. If sizing is performed after annealing, the degree of sulfurisation would fluctuate so that it could become difficult to quantitatively obtain the product. Accordingly, we tried to perform the sizing operation before the annealing step and, as a result, a good result has been obtained. The above-mentioned aspect (1) has a close relation to aspect (2). In accordance with the conventional means of determining a molecular weight by electrophoresis, at least one full night is required for migration, staining and other steps, and therefore, rapid procedure is difficult. In contrast, according to the present invention, HPLC gel-filtration is applied to the determination of the molecular weight of nucleic acid derivatives, whereby the reaction time before the elution of the derivatives having an intended molecular size distribution may be markedly shortened.

In accordance with the present invention, the reaction is stopped after the detection of completion of the sizing reaction, and

then a lower alcohol is added to further process the reaction solution. Among lower alcohols, ethanol is especially preferred.

In the case of ethanol-precipitation by the present invention, for example, the yield of L-poly-C (sized poly-C) (the prefix "L- . ." means "sized . ." hereinafter) from poly-C is 93% and the yield of L-poly-I from poly-I is 78%. Thus the yield of the sized products is high.

In the case of dialysis in the conventional method, the recovery is only 60% or so, and the yield of L-poly-I from poly-I could be expected to be only 40% or so. In addition, dialysis requires 3 days or so to perform.

However, in accordance with the ethanol precipitation method used in the present invention where ethanol is added to a reaction solution in an amount of, say, two times that of the reaction solution and stirred so as to precipitate the desired product and the resulting precipitate is then collected by centrifugation and washed and dried, the process may be finished within one hour.

The above-mentioned aspect (3) is a most important feature.

The reaction of substituting nitrogen atoms in a nucleic acid moiety in a sized single-chain nucleic acid polymer by sulfur atoms (for example, substituting the amino group in the cytidine residue moiety in poly-C by a mercapto group in a certain proportion) so as to convert the said nucleic acid moiety into a different nucleic acid ("sulfurization") is often utilized in the synthesis of copolymers. Another characteristic feature of the present invention is to add an

aryl alcohol to the sized single-chain nucleic acid polymers for isolation of sulfurized copolymers. This is aspect (4) above.

As an aryl alcohol for the purpose, for example, phenol, can be used. For example, a half volume of phenol is first added to the reaction solution containing pyridine, water and hydrogen sulfide gas mixture, stirred and centrifuged, whereby the aqueous layer is definitely separated from the phenol layer and the colouring agent in the reaction solution as well as by-produced sulfur and the like are transferred to the phenol layer. Afterwards, the aqueous layer is isolated and the intended product is precipitated by treatment with an aqueous salt solution and an alcohol. Then the thus precipitated product is isolated by centrifugation and then washed with an alcohol to give a purified product.

In accordance with the said process, almost all the hydrogen sulfide is transferred to the supernatant in the form of a hydrogen sulfide solution and therefore this may easily be removed from the reaction product.

For example, according to the process of the present invention using phenol, the operation may be finished within one hour and the yield is almost 100%. In addition, the product may be isolated quantitatively.

The above-mentioned characteristic aspects (2) and (4) of the present invention are important for improved sizing prior to annealing.

Aspect (5) above is another characteristic feature of the invention, where a step of size-definition is performed between the steps of sizing and annealing. In this step is utilized an ion-exchange resin.

As an example of applying an ion-exchange resin to macromolecular nucleic acids, DEAE-Cellulose, DEAE-Sephadex, benzoylated DEAE-Cellulose or the like is applied to t-RNA (BBA, 47, 193, 1961; BBRC, 10, 200, 1963; Biochem., 6, 3043, 1967).

In the example, however, the ion-exchange resin is applied only to purification of low molecular nucleic acids having a base number of at most 80 or so.

We investigated as to whether the intrinsic property of the charge adsorbability of ion-exchange resins could be applied to purification of macromolecular nucleic acid polymers on the basis of the index of the number of bases of the polymers and, as a result, have established the present invention. Since the final products to be obtained by the present invention are useful as medicines, it is believed that the size-definition to be conducted by the use of an ion-exchange resin ("ion-exchange process") is an important feature in the method of the invention.

In the ion-exchange process of the invention, an ion-exchange resin may be placed in a container containing a nucleic acid polymer to be processed therewith (a batch process), but in general, column chromatography is utilized for fractionation (a column process). Specifically, an ion-exchange resin is placed in a column and a

solution of a nucleic acid polymer is introduced into the column so that the polymer is once adsorbed to the ion-exchange resin. Then an eluant such as salt-tris buffer or the like is linearly or step-wise passed through the column whilst varying the salt concentration so as to obtain a constant amount of an eluate. The number of bases of the nucleic acid polymer as contained in each fraction as eluted is detected by the same HPLC gel-filtration as above using a marker as an index, and accordingly, the fractions containing the intended final product may be collected.

In order to attain the objective of the invention by an ion-exchange process, the nature of the ion-exchange resin and the nature of the eluant are important factors.

For example, when poly-I was dissolved in tris-HCl buffer and was adsorbed to QAE (quaternary aminoethyl) used for size-definition of poly-I, for ion-exchanging, the intended product could not be obtained even though the salt concentration in the eluant was raised to a high level. This is because poly-I itself became insolubilized as the salt concentration in the eluant increased above that of a suitable eluant for poly-I on the QAE resin. This is obvious from the fact that the inosinic acid which is the constitutional unit of poly-I is structurally more hydrophobic. In this case, the salt concentration in a proper eluant for poly-I may be determined in comparison with the case of poly-C.

In another experiment, it was observed that a solution of poly-I became white and cloudy to form a precipitate in a buffer

having a suitable eluant salt concentration for poly-I in a QAE resin. Accordingly, in the ion-exchange process of the present invention, it should be said that the nature of the ion-exchange resin and the eluting salt concentration are important factors.

For example, in the case of poly-I, DEAE resin gave an extremely good result. In the case of poly-C, it was found that both QAE and DEAE resin could give favourable results. For the elution, either salt linear gradient elution of salt stepwise gradient elution can be utilized, whereby the polymers can be fractionated and eluted in the order of the length of the chains of the polymers.

For instance, poly-C (38mg, S_{20}, 8.6) was adsorbed into DEAE-Toyopearl 650 C (0 10 x 130 mm) and then eluted by linear gradient elution using the following eluents (A) and (B) each in an amount of 100 ml.

(A) = 0 M Nacl/10 mM Tris-HCl (pH 7.0)

(B) = 0.5 M NaCl/10 mM Tris-HCl (pH 7.0)

The linear gradient was for (B) (from 0% to 100%); and the elution conditions were as follows:

Linear flow rate: 1.32 cm/min.

Elution rate: 175 drops/fraction.

As a result, the following fractions, each having the chain length indicated, were eluted in order.

Fraction	b.p.
33	340
34	470
35	740
36	1000
37	1500

In addition, the same sample was eluted by stepwise gradient elution, whereby a fraction of lower than 500 b.p. was first eluted with 0.3 M NaCl/10 mM Tris-HCl (pH 7.0) (50 m) and then a fraction of from 500 to 1500 b.p. was eluted with 0.5 M NaCl/10 mM Tris-HCl (pH 7.0) (50 ml).

In the same manner, poly-I (7.8 mg, S_{20}, 7.3) was eluted by linear gradient elution under the same conditions as above. As a result, the following fractions were eluted in order.

Fraction	b.p.
35	30
36	140
37	230
38	350
39	460
40	540

In addition, the same sample was eluted by stepwise gradient elution, whereby a fraction of lower than 300 b.p. was first eluted with 0.3 M NaCl/10 mN Tris-HCl (pH 7.0) (50 ml) and then a fraction of from 300 to 600 b.p. was eluted with 0.5 M NaCl/10 mM Tris-HCl (pH 7.0) (50 ml).

As mentioned above, even macromolecular nucleic acids can be fractionated by the method of the present invention into chain length-defined nucleic acid fractions (size-definition) by appropriately selecting the salt concentration in the elution.

As illustrated in the two above examples, fractions (main components) having a proper chain length distribution suitable for the pharmaceutical property as medicines can be readily fractionated from a mixture containing various macromoleculae nucleic acids with different chain length distributions, and the fractionation can be performed on an industrial mass-production scale. The characteristic in the fractionation is the most important aspect in the ion-exchange process used in the invention.

When a pharmaceutical composition is manufactured in the form of an injection, it is well known that pyrogens should be removed from the injection. The pyrogen is known to be composed of Lipopolysaccharides and should not be incorporated into medical compositions. If the nucleic acid derivatives of the present invention are used as an injection for administration to human, the removal of any pyrogens is essential.

Fortunately, it has been found that use of the ion-exchange process to the nucleic acid derivatives effectively removes pyrogens from the derivatives.

Accordingly, we further continued various experiments so as to more precisely investigate the above-mentioned favourable phenomenon and, as a result, it has further been found that pyrogens

can be removed from any and every single-stranded nucleic acid derivative irrespective of chain length, by the ion-exchange process of the present invention. This is another characteristic aspect of the present invention.

Accordingly, the present invention further provides a method of processing the nucleic acid derivatives of the present invention with an ion-exchange resin so as to remove pyrogens therefrom for preparing an injection containing the derivative.

The results obtained by a limulus test for quantitatively determining the amount of endotoxin in various single-chain RNA's (commercially poly-C and chain-shortened derivatives thereof) are shown in the Table below.

In the Table, EU (endotoxin unit) means a unit in a rabbit fever test with USP reference standard endotoxin (E. coli 0113). (1) indicates distilled water for injection (blank); (2) indicated poly-C (commercial product-1); (3) indicates poly-C (commercial product-2); and (4) indicates the product obtained in the following Example (5-4).

Test Substance	Before or After Ion-exchange Column	Concentration ug/ml	EU/ml	pg/mg	EU/mg
(1)	-	29.92	0.0868		
(2)	Before	308.29	0.8941	548.07	1.5895
	After	18.68	0.0542	33.96	0.0985
(3)	Before	93.89	0.2723	166.18	0.4891
	After	19.45	0.0564	34.12	0.0989
(4)	Before	89.63	0.2599	155.98	0.4520
	After	57.45	0.1666	114.90	0.3332

From the above results, the pyrogen-removing effect by ion-exchange is obvious.

The physiological activity of the nucleic acid derivatives of the present invention makes them useful as medicines. The nucleic acid derivatives of the present invention have a strong carcinostatic activity, discussed hereunder in detail. (This activity is one of various other physiological activities of the nucleic acid derivatives of the present invention.)

Such other physiological activities of the nucleic acid derivatives of the invention which have poly-I·poly-C as the parent body thereof include TNF-producing ability interferon-producing ability, interleukin 2-producing ability, an activity for inhibiting proliferation of tumor cells, an activity for inhibiting proliferation of tumor cells in human tumor cell-bearing nude mice, and activity of inhibiting metastasis of tumor cells in the lung.

The nucleic acid derivatives of the invention have higher safety that conventional poly-I·poly-C and the like interferon-inducers. Accordingly, the compounds of the invention are useful as antiviral agents, antitumor agents, etc.

The physiological activities of the nucleic acid derivatives of the present invention as mentioned above as described in detail in the aforesaid patent, Japanese Patent application No. 62-167433 and the co-pending application claiming priority therefrom.

In order that the invention may be well understood, the following Example is given by way of illustration only.

EXAMPLE

(1) Preparation and purification of L-poly-I (sized Poly-I):

200 ml of a distilled water, 250 ml of formamide and 500 ml of a 5M NaCl solution were added to 10 g of a commercial poly-I and heated at 80°C for about 4 hours.

The reaction solution was subjected to gel-filtration by HPLC using TSK gel G-DNA-PW column (7.88 mm ID x 300 mm) (eluent: 50 mM Tris-HCl buffer (pH 7.5), 0.3 M NaCl solution, 2mM EDTA; flow rate: 0.5 ml/min), whereupon the reaction was stopped at the time when a fraction with a maximum peak for a retention time of 21.86± 0.2 minutes was obtained.

A twice amount of ethanol was added to the reaction solution and the resulting precipitate formed was collected by centrifugation (3000 rpm, 4°C). This was washed with 70 % ethanol and dried in a vacuum to obtain 10.2 g of L-poly-I.

Water and the solutions used in the above process were all sterilized ones. The same shall apply hereinafter.

(2) Preparation and purification of L-poly-C (sized poly-C):

200 ml of a distilled water, 250 ml of formamide and 50 ml of a 5M NaCl solution were added to 10 g of poly-C and heated at 80°C for about 4 hours. By the same HPLC gel-filtration as above, the end point of the reaction was confirmed (retention time: 21.33 ± 0.2 min).

A twice amount of ethanol was added to the reaction solution and the precipitate formed was collected by centrifugation

(3000 rpm, 4°C). This was washed with 70 % ethnaol and then dried in a vacuum to obtain 9.5 g of L-poly-C.

(3) Sulfurization of L-poly-C:

8.0 g of the L-poly-C obtained in the above step (2) was dissolved in 240 ml of water and put in a 500 ml steel bomb. A pyridine solution containing hydrogen sulfide (12 g/120 ml) was added thereto with ice-cooling. After sealed, the bomb was heated at 50°C for about 10 hours. After cooled, a TE-saturated phenol (200 ml) was added, stirred and centrifuged (3000 rpm, 15°C, 5 minutes). A 1/10 amount of a 5M NaCl solution and a twice amount of ethanol were added to the aqueous water as separated to give a precipitate therein. The precipitate was collected by centrifugation (3000 rpm, 4°C, 10 minutes), washed with 70 % ethanol and dried in a vacuum to give 8.0 g of L-poly(C_{20}, S^4 U) (namely, sized poly-C derivative in which the cytidylic acids are substituted by 4-thiouridylic acid in an amoumt of one 4-thiouridylic acid for 20 cytidylic acids).

The above mentioned TE means 10 mM Tris-HCl buffer (pH 7.5) containing EDTA in an amount of 1 mM.

(4) Embodiment of Annealing:

6.00 g of the L-poly(C_{20}, S^4 U) obtained in the above-mentioned step (3) and 6.44 g of the poly-I obtained in the above-mentioned step (1) were dissolved in 300 ml of 10 mM Tris-HCl buffer (pH.7.5)/50 mM NaCl solution and blended therein. The resulting solution was heated up to 70°C in a water bath and

kept at the said temperature for 10 minutes. Afterwards, this was left to be cooled overnight as it was. After phenol-treatment and ethanol-precipitation, water (about 200 ml) was added to the precipitate formed so as to dissolve it. Then the resulting solution was dialyzed against water at 4°C. The dialysate was concentrated to dryness to give 12.4 g of an annealed compound.

(5) Size-definition by Ion-exchange Process:

The ion-exchange was conducted by stepwise elution or linear gradient elution. In both cases, the yield and the chain length of the products were almost the same, provided that the elution condition was properly selected. For the stepwise elution of L-poly-C and L-poly(C, S^4U), 0.15 M NaCl/10 mM Tris-HCl (pH 7.0) and 1.0M NaCl/10 mM Tris-HCl (pH 7.0) were used continuously.

Regarding the stepwise elution of L-poly-I, Example (5-1) to follow may be referred to.

For the linear gradiation elution of L-poly-I, the following (A) and (B) were used, and the elution was conducted under the gradient condition of the solution (B) of from 0 to 100 %.

(A) = 0 M NaCl/10 mM Tris-HCl (pH 7.0)

(B) = 0.5 M NaCl/10 mM Tris-HCl (pH 7.0).

Regarding the linear gradiation elution of L-poly-C and L-poly(C, S^4 U), Example (5-2) and (5-4) to follow may be referred to.

(5-1) Size-definition of L-poly-I:

210 mg of the L-poly-I obtained in the above-mentioned step (1) was dissolved in 5 ml of 10 mM Tris-HCl buffer (pH 7.0) and adsorbed to DEAE-Toyopearl^{RTM} 650 C (ϕ 10 mm x 130 mm). Then this was stepwise eluted at a linear flow rate of 1.30 cm/min, with eluents of 0.03 M NaCl/10 mM Tris-HCl (pH 7.0) (50 ml) and 0.5 M NaCl/10 mM Tris-HCl (pH 7.0) (80 ml). The fraction eluted with 0.5 M NaCl was collected and the retention time thereof was measured by the same HPLC gel-filtration as in the above-mentioned step (1), which was 21.90± 0.2 (min).

The intended final product L-poly-I (size-defined) having a base number of from 100 to 1000 was obtained with a high yield. The recovery yield was 91 %.

(5-2) Size-definition of L-poly-C:

610 mg of the L-poly-C obtained in the above-mentioned step (2) was dissolved in 10 ml of Tris-HCl buffer (pH 7.0) and adsorbed to QAE-Toyopearl^{RTM} 550 C (ϕ 10 mm x 130 mm). Then this was eluted by linear gradient elution at a linear flow rate of 1.30 cm/min, whereupon the following (A) and (B) were used each in an amount of 100 ml and the elution was conducted under the gradient condition of the solution (B) of from 0 ot 100 %.

(A) = 0.0 M NaCl/10 mM Tris-HCl (pH 7.0)

(B) = 1.0 M NaCl/10 mM Tris-HCl (pH 7.0).

The fraction as eluted at th main peak was collected and the retention time thereof was measured by HPLC gel-filtration, which was 21.35±0.2 min. The intended final product L-poly-C (size-defined) having a base number of from 100 to 1000 was obtained with a high yield. The recovery yield was 93 %.

(5-3) Size-definition of L-poly(C_{12}, U):

19 mg of poly(C_{12}, U) (in which the cytidylic acids were substituted by uridylic acid in an amount of one uridylic acid for 12 cytidylic acids) (this had a retention time of 18.67 min in the same HPLC as in the above step (1)) was dissolved in 5 ml of Tris-HCl buffer (pH 7.0) and adsorbed to DEAE-Toyopearl^{RTM} 650 C (ϕ10 mm x 130 mm). Then this was eluted by linear gradient eluiton at a linear flow rate 1.30 cm/min, whereupon the following (A) and (B) were used each in an amount of 100 ml and the elution was conducted under the gradient condition of the solution (B) of from 0 to 100 %.

(A) = 0.0 M NaCl/10 mM Tris-HCl (pH 7.0)

(B) = 0.5 M NaCl/10 mM Tris-HCl (pH 7.0)

The fraction as eluted at the main peak was collected and the retention time thereof was measured by HPLC gel-filtration, which was 18.97±0.2 min. The intended final product L-poly-(C_{12}, U) (size-defined) having a base number of from 100 to 1000 was obtained with a high yield. The recovery yield was 87 %.

(5-4) Size-definition of L-poly(C_{20}, S^4 U):

600 mg of the L-poly(C_{20}, S^4 U) obtained in the above-mentioned step (3) was dissolved in 10 ml of Tris-Hcl buffer (pH 7.0) and adsorbed to QAE-Toyopearl^{RTM} 550 C (10 mm x 130 mm). Then this was eluted by linear gradient elution at a linear flow rate cm/min, whereupon the following (A) and (B) were used each in an amount of 100 ml and the elution was conducted under the gradient condition of the solution (B) of from 0 to 100 %.

(A) = 0.0 M NaCl/10 mM Tris-HCl (pH 7.0)

(B) = 1.0 M NaCl/10mM Tris-HCl (pH 7.0)

The retention time was measured by HPLC gel-filtration in the same manner as in the above-mentioned step (5-2), which was 21.35± 0.2 min.

The intended final product L-poly(C_{20}, S^4 U) (size-defined) having a base number of from 100 to 1000 was obtained with a high yield. The recovery yield was 90 %.

(6) Annealing:

(6-1) L-poly-I and L-poly-C:

3.0 g of the size-defined L-poly-C (obtained in the above step (5-2)) and 3.2 g of the size-defined L-poly-I (obtained in the above step (5-1)) were dissolved separately in 150 ml of 10 mM Tris-HCl buffer (pH 7.5)/50 mM NaCl and they were blended. The resulting solution was heated up to 70°C in a water bath and was kept at the said temperature for 10 minutes. Afterwards, this

was left to be cooled overnight as it was. After phenol-treatment and ethanol-precipitation, water (about 400 ml) was added to the precipitate formed so as to dissolve it. Then the resulting solution was dialyzed against water at 4°C. The dialysate was concentrated to dryness to obtain 6.2 g of an annealed compound.

(6-2) L-poly-I and L-poly-(C_{20}, S^4 U):

1.46 g of the size-defined L-poly(C_{20}, S^4 U) (obtained in the above step (5-4)) and 1.57 g of the size-defined L-poly-I (obtained in the above step (5-1)) were processed in the same manner as in the above-mentioned step (6-1), and 3.0 g of an annealed compound was obtained in each case.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Claims:

1. A method of producing double-stranded nucleic acid derivatives having RNA as the parent body, the total molecular size distribution of which is from 4S to 13S expressed as sedimentation constant value, in which nucleic acid polymers are sized and then annealed.

2. A method of producing double-stranded nucleic acid derivatives having RNA as the parent body, in which the molecules for the maximum distribution in the total molecular size distribution of the derivatives have a base number of from 50 to 10,000, in which nucleic acid polymers are sized and then annealed.

3. A method as claimed in Claim 1 or Claim 2, in which a lower alcohol is added to the sized nucleic acid polymer-containing reaction solution before annealing.

4. A method as claimed in Claim 3 in which the lower alcohol is ethanol.

5. A method as claimed in any one of the preceding Claims in which the nucleic acid moieties in the sized single-stranded nucleic acid polymers are sulfurized in the presence of an aryl alcohol, before annealing.

6. A method as claimed in Claim 5, in which the aryl alcohol is a phenol.

7. A method as claimed in any of the preceding Claims in which the sized single-stranded nucleic acid polymers are processed with an ion-exchange resin, before annealing, so as to collect polymers with

a molecular size distribution falling within a determined range for size-definition thereof.

8. A method of preparing an injection consisting essentially of nucleic acid derivatives, characterized in that pyrogens in the derivatives, if any, are removed by the use of an ion-exchange resin.

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